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Rapid O serogroup identification of the six clinically relevant Shiga toxin-producing *Escherichia coli* by antibody microarray

Narasimha V. Hegde [a,*], Craig Praul [b], Andrew Gehring [c], Pina Fratamico [c], Chitrita DebRoy [a]

[a] *Escherichia coli Reference Center, Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, PA 16802, USA*
[b] *Genomics Core Facility, Pennsylvania State University, University Park, PA 16802, USA*
[c] *USDA, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA 19038, USA*

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ABSTRACT

An antibody microarray was developed to detect the "top six" non-O157 serogroups, O26, O45, O103, O111, O121, and O145 of Shiga toxin-producing *Escherichia coli* (STEC), that have been declared as adulterant in meat by the Food Safety and Inspection Service of the United States Department of Agriculture. The sensitivity of the array was 10^5 CFU and the limit of detection of each serogroup in artificially inoculated ground beef was 1–10 CFU following 12 h of enrichment. Optimal concentrations of antibodies for printing and labeling and bacterial dilutions for binding to the antibodies were assessed. The array utilized a minimal amount of antibodies and other reagents and may be utilized for screening of multiple target O groups of STEC in parallel, directly from enriched samples in less than 3 h. Furthermore, the antibody array provides the flexibility to include other O serogroups of *E. coli* and may be adopted for high throughput screening. The method is potentially applicable to detect the pathogenic STEC O groups of *E. coli* in meat and other food, thus improving food safety and public health.

1. Introduction

Over the last decade, there is a growing concern for gradual increase in the occurrence of foodborne illnesses due to non-O157 Shiga toxin-producing *Escherichia coli* (STEC). The U.S. Department of Agriculture estimates 70–80% of non-O157 STEC related illnesses have been associated with serogroups O26, O45, O103, O111, O121, O145 (USDA-FSIS, 2011a) and therefore, these six non-O157 STEC serogroups have been declared as adulterants in beef by the Food Safety and Inspection Services (FSIS) of the USDA who recently enforced a new policy for testing these O groups in beef. Conventional method for *E. coli* O serogroup determination relies on agglutination reaction when denatured *E. coli* that are heated at 100 °C for 2 h are mixed with specific antibodies generated against the O antigen. Serotyping is laborious and sometimes exhibit equivocal results. To overcome this difficulty, detection of the unique *wzx* genes of the O antigen gene clusters of the six STEC O groups by polymerase chain reaction (Fratamico et al., 2011; DebRoy et al., 2011) and luminex microbead-based suspension arrays (Lin et al., 2011) have been developed. Recently, we have reported rapid and sensitive assays for the detection of the top six non-O157 STEC O serogroups using ELISA and flow cytometry (Hegde et al., 2012a, 2012b). ELISA, a colorimetric assay, lacks the ability to detect multiple target serogroups in a single reaction that could be accomplished by flow cytometry by labeling antibodies with different fluorophores. However, flow cytometry is not economical for routine screening. Antibody arrays have been reported for simultaneous detection of *E. coli* O157:H7 and *Salmonella spp* (Karoonuthaisiri et al., 2009), a quantum dot-based array was developed for the detection of *Escherichia coli* O157:H7 (Sanvicens et al., 2011), carbohydrate antigen microarrays were established to detect *Salmonella* O-antigen specific antibodies (Blixt et al., 2008), and antibody microarray in multi-well plate format was utilized for multiplex screening of foodborne pathogens (Gehring et al., 2006, 2008). Although our ability to detect polyclonal outbreaks has improved considerably, there is a greater potential for antibody microarray to be utilized for the detection of different pathogens or serogroups for improving food safety. The aim of this study was to develop an antibody microarray for simultaneous detection of top six non-O157 STEC O serogroups that can be adopted for clinical and food safety testing.

2. Materials and methods

2.1. Antibodies

Purified polyclonal anti-rabbit antibodies generated against reference *E. coli* strains belonging to serogroups O26, O45, O103, O111, O121, and O145 were obtained from SDIX (Newark, DE). Specificities of these antibodies were tested against reference strains (from WHO) belonging to serogroups O1 through O181, ten clinical isolates confirmed to belong to each of the six non-O157 STEC O groups (n = 60 isolates) and ten other bacterial species such as *Citrobacter freundii,*

* Corresponding author. Tel.: +1 814 863 2167; fax: +1 814 867 2096.
E-mail address: nvh1@psu.edu (N.V. Hegde).

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Enterobacter cloacae, Hafnia alvei, Klebsiella pneumoniae, Proteus vulgaris, Salmonella enterica serovars Enteritidis and Typhi, *Serratia marcescens, Shigella boydii, and Shigella flexneri* as previously described (Hegde et al., 2012b).

2.2. Sample preparation for printing on the array

Purified antibodies for each serogroup (0.15 μg/μl) in phosphate-buffered saline (PBS) were mixed with an equal volume of 2x protein printing buffer (Arrayit, Sunnyvale, CA). Reference *E. coli* strains H311b (O26), K42 (O45), H515b (O103), Stoke W (O111), 39w (O121), E1385 (O145) were grown in Tryptic Soy Broth (TSB) for 16 h at 37 °C with shaking. Bacterial cells were harvested and resuspended in 1 ml of PBS at 2×10^6 CFU/ml and boiled for 1 h at 100 °C. The heat inactivated cells were centrifuged at 12,000 × *g* for 10 min. The supernatant (20 μl) containing the O antigen polysaccharides (positive control) were mixed with equal volume of printing buffer. Rabbit IgG (Sigma-Aldrich, St. Louis, MO) in printing buffer (0.075 μg/μl final concentration) was also printed on the array (negative control).

2.3. Printing arrays

Microarrays were printed at the Pennsylvania State University Genomics Core Facility. A 15 × 6 spot matrix in which five spots for each of the six antibodies, five spots for rabbit IgG, and five spots for respective polysaccharide were printed on SuperEpoxy 2 (Arrayit, Sunnyvale, CA) slides using a OmniGrid microarrayer (GeneMachines, San Carlos, CA). Printing was performed at 22 °C with 50% humidity using SMT-S50 pins (Parallel Synthesis Technologies, Santa Clara, CA).

2.4. Antibody labeling

Antibodies against the six O groups were labeled using the Zenon Rabbit IgG labeling kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Labeling mixture (10 μl) comprising of antibodies (0.2 μg) and 0.5 μl group of Zenon rabbit IgG labeling reagent (Alexa Fluor® 555) was incubated for 5 min at room temperature (RT). Zenon blocking reagent 0.5 μl was added and incubated for an additional 5 min at RT, and finally 40 μl of PBS was added to the reaction mixture and used for one subarray. When a mixture of antibodies against multiple serogroups were to be labeled in a single reaction, the concentrations of respective antibodies (0.2 μg), labeling reagent (0.5 μl/O group) and blocking reagent (0.5 μl/0.5 μl of labeling reagent) were proportionately increased.

2.5. Sample preparation for testing on the array

The reference *E. coli* strains belonging to serogroups O26, O45, O103, O111, O121, and O145 were grown in 5 ml TSB overnight at 37 °C. The cell density of the cultures was adjusted to 10^7, 10^6, 10^5, 10^4 CFU/100 μl in PBS for determining the limit of detection (LOD) on the microarray. Cell numbers were also confirmed by the aerobic plate count method. The reference *E. coli* strains belonging to the six serogroups were spiked individually or two or three serogroups were simultaneously inoculated in ground beef (162.5 g ground beef in 487.5 ml TSB) at 1–10 CFU/serogroup. The cultures were enriched for 12 h following USDA recommended protocol (USDA-FSIS, 2011b). Un-inoculated ground beef samples were processed similarly that served as a negative control as well as for assessing the background signals. Following enrichment, the sample (1 ml) was centrifuged at 1000 × *g* for 1 min to remove large debris and the supernatant was transferred to a fresh tube. The cells were centrifuged at 12,000 × *g* for 5 min to collect bacterial cells. Cell pellet was re-suspended in 1 ml PBS by pipetting five to six times and cells were centrifuged at 12,000 × g for 5 min. This step was repeated one more time and finally bacterial cells were re-suspended in 100 μl of PBS and applied onto the each subarray.

2.6. Cell hybridization and detection

Glass slides containing the arrays were mounted on a hybridization cassette, and 200 μl of BlockIt microarray blocking buffer (Arrayit) containing 5% BSA (Sigma-Aldrich) were added to each array and incubated for 1 h at RT. The blocking solution was then removed, and 100 μl of PBS containing different dilutions of pure culture or after enrichment was added to each subarray and incubated for 1 h at RT. The cells were removed, and the array was washed five times (1 min each wash) with PBS (200 μl) containing 0.05% Tween 20 (PBST). Labeled antibodies were applied on the array and incubated (protected from direct light) for 45 min at RT. The slide was removed from the hybridization cassette and washed in PBST (25 ml) for 15 min, dried by centrifugation at 1000 × *g* for 2 min, and scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). For each cell dilution, the fluorescence intensity was expressed in relative units as the average of 5 spots in each array and standard deviation was calculated from three replicate arrays. Fluorescence intensities obtained from labeled antibodies without the addition of target cells in an array was used to determine the background fluorescence intensity. Differences in the signal intensities were compared using an unpaired *t* test and considered significant at $p < 0.05$.

3. Results and discussion

Serogroup specific antibodies were printed on the epoxy coated slide in a microarray to capture target STEC O groups. Captured cells were further detected by fluorescent-labeled antibodies. Respective polysaccharide antigens produced by heating the bacteria at 100 °C for 2 h were printed on the slide that served as in-built positive controls assessing the labeling efficiency. Since the antibodies were raised in rabbits, rabbit IgG printed on the slide served as the negative controls in the microarray. A schematic representation of the placement of the antibodies on the array is presented in Fig. 2A.

The LOD, for the top six STEC O serogroups was determined by applying different concentrations (10^4 to 10^7 CFU) of *E coli* reference strains on an individual subarray. While microarray at 10^4 CFU was found to be insufficient to provide reliable fluorescence intensities (RFU) above the background, concentrations at 10^5 CFU and above consistently provided RFU above the background. The median fluorescence intensities obtained after subtracting background intensities at 10^5 CFU for all six serogroups are depicted. (Fig. 1, $p < 0.05$). The experiment was repeated five times and RFU between subarrays were consistent in different slides with no nonspecific binding detected. The RFU values for the six serogroups at 10^5 CFU varied between 12,600 and 56,250 and the background RFU values were found to be between 2086 and 3760 RFU (Fig. 1). Antibodies to serogroup O121 exhibited the highest signal followed by O145, O111, O26, O103, and O45 (Fig. 1). Since each O antigen polysaccharide is unique, differences in the signal intensity may be due to several factors such as antibody titer, antigen-antibody binding constant, and antibody labeling efficiency.

To test whether antibody array can be used to detect specific *E. coli* O serogroups enriched in ground beef, individual target O serogroup was artificially inoculated (1–10 CFU) in ground beef, enriched for 12 h as described, and tested on the array. As depicted in Fig. 2, no cross reactions between the O groups was observed and all target O groups were identified (Fig. 2B). Initially one target O group was spiked and enriched in ground beef and tested on the array with labeled antibodies to respective target serogroup as shown in Fig. 2B. Subsequently, two serogroups (O26 and O145) were spiked and enriched simultaneously in the beef sample and were detected on the array by labeling antibodies against O26 and O145 in a single

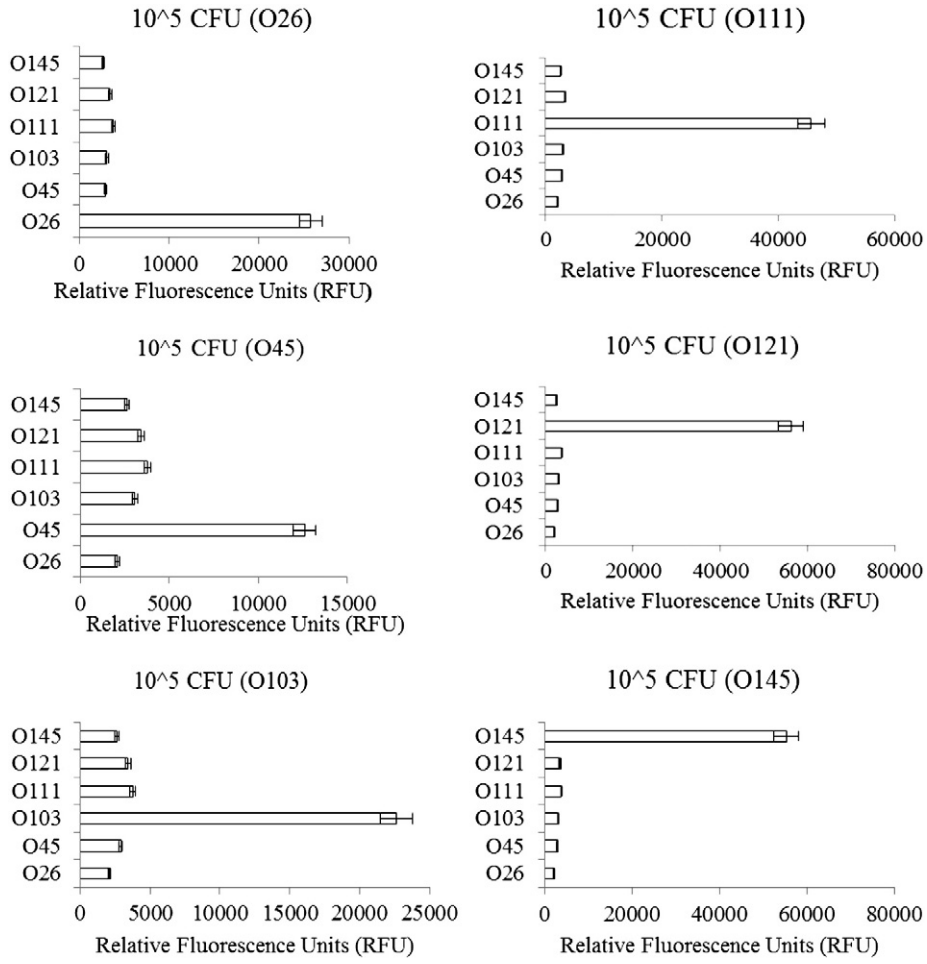

Fig. 1. Relative fluorescence units (RFU) for each target serogroup at 10^5 CFU. Array was gridded, and for each serogroup on the array, averages of median fluorescence intensity from five spots are presented. Experiment was repeated five times. Differences between arrays were tested for statistical significance by using *t*-test probabilities (significance at <0.05).

labeling reaction (Fig. 2C (a)). To determine the specificity of the reaction, two serogroups (O121 and O145) were spiked and enriched simultaneously in the beef sample and detected on the array by a mixture of antibodies to all six serogroups, that were labeled in a single labeling reaction (Fig. 2C (c)). Only the two target serogroups (O121 and O145) were captured by the antibodies on the array thus exhibiting specificity of the reaction. As a positive control, the polysaccharides of six O groups printed on the array reacted positively to the mixture of six antibodies. Similarly, in Fig. 2C (b), three serogroup (O26, O45, and O103) were spiked and enriched simultaneously in a beef sample and detected on the array by a mixture of labeled antibodies to these three target serogroups. In Fig. 2C (d), the three target serogroups (O26, O45, and O121) were spiked and enriched simultaneously in a beef sample and detected on the array by a mixture of labeled antibodies to six serogroups that included three non-target O groups. Un-inoculated beef samples that served as a negative control exhibited bacterial growth (background flora) during enrichment, but did not react with the antibodies printed on the array showing the specificity of the assay. The labeled antibodies bound only to the respective polysaccharides printed on the array indicating the absence of the target serogroup in this enriched diverse bacterial population (Fig. 2C (e)). In the real world samples, we can expect the presence of *E. coli* belonging to any one or more serogroups following enrichment. Enriched samples can be tested for all six serogroups simultaneously by adding six antibodies to a single labeling reaction.

Different concentrations of antibodies were printed on the glass slide to test for its ability to capture bacteria. Antibody concentration of 0.075 μg/μl was found to be optimum for capturing sufficient number of cells and to provide minimum background RFU. PBS containing 5% BSA and commercially available blocking reagent (BlockIt microarray blocking buffer) were compared during the blocking step and BlockIt buffer reduced the background fluorescence intensities. Zenon labeling technology that utilizes a fluorophore labeled Fab fragment directed against the Fc portion of the IgG (host specific) was adopted to label six antibodies in a single reaction. Antibody array technique may be utilized for simultaneous detection of multiple targets in a single reaction, with much shorter assay time that may be potentially exploited for testing multiple foodborne pathogens simultaneously. One of the advantages of the assay would be the preservation of the sample after enrichment that may be used for isolation and confirmation as mandated by FSIS. After enrichment step, samples can be preserved or transported to a central screening facility where in assays can be completed in less than three hours.

4. Conclusions

Antibody microarray was developed that could detect top six non-O157 STEC O serogroups in artificially inoculated ground beef. The array was found to be sensitive, specific and reproducible. The microarray developed and tested on epoxy glass slides can be adapted to a multi-well plate format to provide a greater flexibility for high

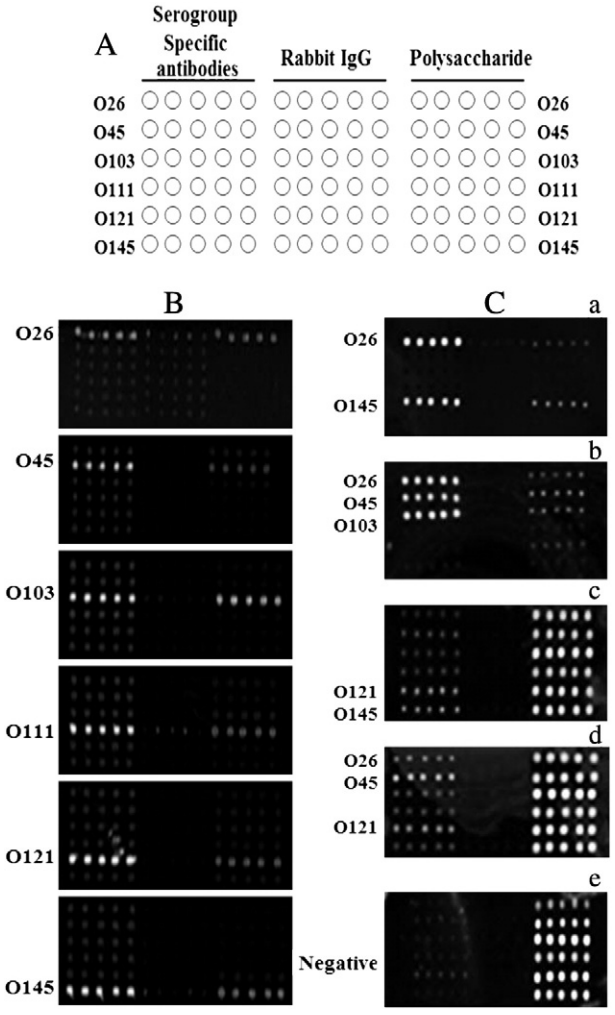

Fig. 2. Antibody subarray design and detection of O serogroups spiked in ground beef samples following 12 h enrichment. [A], 15 × 6 spot matrix, a schematic representation of the placement of antibodies and polysaccharides on the microarray. [B], Scanned images of subarrays showing the detection of individual O serogroups. C (a), two serogroups (O26 and O145) were spiked and enriched simultaneously in the beef sample and detected on the array by mixture of labeled antibodies against O26 and O145 serogroups. C (b), detection of three target O serogroups (O26, O45, and O103) following enrichment in beef sample by a mixture of labeled antibodies to target serogroups. C (c), detection of two target O serogroups (O121 and O145) on the array by a mixture of six antibodies labeled in a single reaction. C (d), a mixture of six labeled antibodies was used for the detection of three target O groups spiked and enriched in ground beef sample. C (e), Un-inoculated ground beef samples enriched (negative control) and tested on the array using a mixture of six antibodies labeled in a single reaction. Positive signals only from polysaccharides, and no signal from rabbit IgG or serogroup specific antibodies printed on the array.

throughput screening. The antibody microarray is potentially useful for developing target specific antibody/antigen chips for antibody screening or for simultaneous detection of clinically relevant pathogens.

References

Blixt, O., Hoffmann, J., Svenson, S., Norberg, T., 2008. Pathogen specific carbohydrate antigen microarrays: a chip for detection of *Salmonella* O-antigen specific antibodies. Glycoconj. J. 25, 27–36.

DebRoy, C., Roberts, E., Valadez, A.M., Dudley, E.G., Cutter, C.N., 2011. Detection of Shiga-toxin producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex PCR of the *wzx* gene of the O-antigen gene cluster. Foodborne Pathog. Dis. 8, 651–652.

Fratamico, P.M., Bagi, L.K., Cray Jr., W.C., Narang, N., Yan, X., Medina, M., Liu, Y., 2011. Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin–producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 in ground beef. Foodborne Pathog. Dis. 8, 601–607.

Gehring, A.G., Albin, D.M., Bhunia, A.K., Reed, S.A., Tu, S.I., Uknalis, J., 2006. Antibody microarray detection of *Escherichia coli* O157:H7: quantification, assay limitations, and capture efficiency. Anal. Chem. 78, 6601–6607.

Gehring, A.G., Albin, D.M., Reed, S.A., Tu, S.I., Brewster, J.D., 2008. An antibody microarray, in multiwall plate format, for multiplex screening of foodborne pathogenic bacteria and biomolecules. Anal. Bioanal. Chem. 391, 497–506.

Hegde, N.V., Jayarao, B.M., DebRoy, C., 2012a. Rapid detection of top six non-O157 Shiga toxin-producing *Escherichia coli* O groups in ground beef by flow cytometry. J. Clin. Microbiol. 50, 2137–2139.

Hegde, N.V., Cote, R., Jayarao, B.M., Muldoon, M., Lindpaintner, K., Kapur, V., DebRoy, C., 2012b. Detection of the top six non-O157 Shiga toxin producing *Escherichia coli* O groups by ELISA. Foodborne Pathog. Dis. 9, 1044–1048.

Karoonuthaisiri, N., Charlermroj, R., Uawisetwathana, U., Luxananil, P., Kirtikara, K., Gajanandana, O., 2009. Development of antibody array for simultaneous detection of foodborne pathogens. Biosens. Bioelectron. 24, 1641–1648.

Lin, A., Nguyen, L., Lee, T., Clotilde, L.M., Kase, J.A., Son, I., Carter, M.J., Lauzon, C.R., 2011. Rapid O serogroup identification of the ten most clinically relevant STECs by Luminex microbead-based suspension array. J. Microbiol. Methods 87, 105–110.

Sanvicens, N., Pascual, N., Fernández-Argüelles, M.T., Adrián, J., Costa-Fernández, J.M., Sánchez-Baeza, F., Sanz-Medel, A., Marco, M.P., 2011. Quantum dot-based array for sensitive detection of *Escherichia coli*. Anal. Bioanal. Chem. 399, 2755–2762.

USDA-FSIS, 2011a. Risk profile for pathogenic non-O157 Shiga toxin-producing *Escherichia coli* (non-O157 STEC). http://www.fsis.usda.gov/pdf/non_o157_stec_risk_profile.pdf.

USDA-FSIS, 2011b. Detection and Isolation of non-O157 Shiga toxin- producing *Escherichia coli* (STEC) from meat products (MLG 5B.01). http://www.fsis.usda.gov/PDF/MLG-5B.pdf.